# Inhibition of Mushroom Polyphenoloxidase by Ascorbic Acid Derivatives

A.F. HSU, J.J. SHIEH, D.D. BILLS, and K. WHITE


## ABSTRACT

Ascorbic acid (AA), dehydroascorbic acid (dehydroAA), isoascorbic acid (isoAA), ascorbic acid-2-phosphate (AA-2- $PO_4$), and ascorbic acid-2-sulfate (AA-2-$SO_4$) were tested as inhibitors of mushroom polyphenoloxidase (PPO). Kinetic analysis indicated that AA and isoAA were more effective than dehydroAA. The half times ($t_{1/2}$) that decreased 50% of PPO activity for AA, isoAA and dehydroAA were 2.5, 3.1, and 1.9 hr, respectively, and the concentrations that inhibited half of PPO activity were as follows: ascorbic acid, 0.04 mM, isoAA, 0.25 mM; and dehydroAA, 7.5 mM. Electron spin resonance studies demonstrated that the $Cu^{2+}$ of PPO was reduced to $Cu^{+}$ by AA. AA-2-$PO_4$ and AA-2-$SO_4$ were not inhibitors for PPO. However, the digestion of AA-2-$PO_4$ with acid phosphatase yielded AA to inhibit PPO activity. AA-2-$SO_4$ was found to be a poor substrate for sulfatase.


## INTRODUCTION

ENZYMATIC BROWNING of raw fruits and vegetables is caused mainly by the conversion of native phenolic compounds to quinones, which are polymerized, in turn, to brown, red or black pigments. The enzymes responsible for the sequence of reactions are usually referred to as polyphenoloxidases [E.C. 10.3.10.1; diphenol: $O_2$ oxidoreductase; polyphenoloxidase (PPO)], but are also known as tyrosinases, catecholases, cresolases, and phenolases (Mason, 1955, 1965; Kertesz and Zito, 1962; Mathew and Parpia, 1971; Mayer and Harel, 1979). Browning occurs when the phenolic substrates, the PPO enzymes, and oxygen are brought together under appropriate conditions of pH, temperature, and water activity. Bruising, cutting, peelings or otherwise disrupting cells promotes enzymatic browning of many fruits and vegetables. Senescence or disease also can lead to enzymatic browning. For fresh or processed fruits and vegetables, the enzymatic browning produces undesirable colors and off-flavors (Scott, 1975). In addition to the loss of aesthetic quality of fruits and vegetables, enzymatic browning also reduces nutritional quality through the destruction of nutrients such as ascorbic acid (Mathew and Parpia, 1971; Synge, 1975).

Because of the deleterious effect of enzymatic browning on fruits and vegetables, much work has been devoted to the development of methods for eliminating or at least retarding the process. Theoretically, there are several approaches to the problem, but of greatest current practical significance to the fruit and vegetable industry are methods that depend on the reduction of quinones formed by the oxidation of phenols by PPO or the inhibition or inactivation of PPO. Reducing agents, such as ascorbic acid, cysteine and sulfur dioxide, reduce the *o*-quinones to their *o*-phenol precursors (Eskin et al., 1971; Synge, 1975; Walker, 1977). However, the effect of such reducing agents is temporary, because they themselves are irreversibly oxidized during the process. The use of reducing agents also can lead to oxidation products with off-flavors. The use of a commonly used reducing agent, sulfite, is the subject of current re-examination because of public health questions. At high levels, naturally occurring sulfhydryl compounds, such as cysteine, can react with *o*-quinones to form colorless thioethers (Walker, 1977). There are only a few circumstances where these compounds are of any value in food systems.

Ascorbic acid is widely used to inhibit enzymatic browning of fruits and vegetables because it is a nontoxic compound at levels employed. The inhibition of PPO by ascorbic acid is complex. Inhibition of brown color formation in the reaction of mushroom PPO and o-dihydroxyphenols has been demonstrated by Golan-Goldhirsh and Whitaker (1984). The mechanism of ascorbic acid inhibition has generally been attributed to the reduction of o-quinone back to the phenolic substrate. Varoquaux and Sarris (1979) suggested that ascorbic acid neither inhibits nor activates the enzyme. In contrast to this, other reports (Baruah and Swain, 1953; Ponting, 1954) suggested a direct interaction between ascorbic acid and PPO. The mechanism of inactivation is not clear from these results. A K-type interaction of PPO with ascorbic acid was reported (Golan-Goldhirsh and Whitaker, 1985). With this type of inhibition, the product of the reactions reacts with the enzyme to form a covalent enzyme derivative which is inactive.

Although ascorbic acid is the first choice for anti-browning in fresh fruits and vegetables, its instability led us to further investigate other ascorbic acid derivatives that may have greater stability and increased effectiveness in preventing browning. Two AA derivatives, AA-2-$PO_4$ and AA-2-$SO_4$, were of most interest, since the time required for enzymatic conversion of AA-2-$PO_4$ or AA-2-$SO_4$ to AA may act as a pulse releasor for AA to inhibit PPO.

In the course of these investigations, Electron Paramagnetic Resonance Spectroscopy (EPR) was utilized to establish the

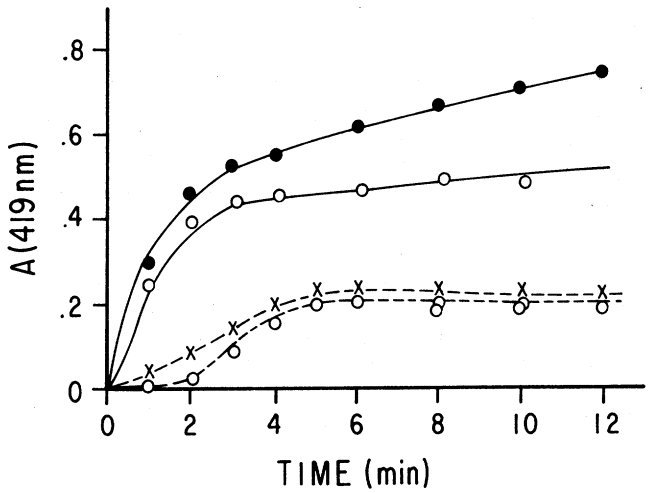


*Fig. 1—Comparison of ascorbic acid derivatives on inhibition of mushroom PPO. AA (○- - -○, isoAA (x—x), dehydroAA (○—○), AA-2-$PO_4$ (●—●) and AA-2-$SO_4$ (●—●) at 0.25 mM concentration were incubated with 20 μg of mushroom PPO and 0.25 mM DOPA in 50 mM sodium phosphate buffer (pH 6.8) at 25°C. The control experiment (●—●) was carried out without above inhibitors under the same conditions. The activity of PPO was measured spectrophotometrically at 419 nm.*

*The authors are with the USDA-ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118.*

Table 1—Comparison of different inhibitors of enzymatic browning on a system containing DOPA and mushroom polyphenoloxidase

| Variable[b] | Inhibitor[a] AA | ISoAA | DehydroAA | AA-2-$PO_4$ | AA-2-$SO_4$ |
|---|---|---|---|---|---|
| $t_{1/2}$ (hr) | 2.50 ± 0.10[c] | 3.10 ± 0.11 | 1.90 ± 0.10 | N[d] | N |
| $I_{1/2}$ (mM) | 0.040 ± 0.002 | 0.250 ± 0.001 | 7.500 ± 0.370 | N | N |

[a] AA = Ascorbic acid, IsoAA = Isoascorbic acid, DehydroAA = Dehydroascorbic acid, AA-2-$PO_4$ = Ascorbic-2-Phosphate, AA-2-$SO_4$ = Ascorbic-2-Sulfate.
[b] $t_{1/2}$ = Preincubation time (time before DOPA substrate added to the system) required for 0.25 mM inhibitor to reduce the activity of mushroom polyphenoloxidase by 50%. $I_{1/2}$ = The concentration of inhibitor required to decrease the amount of browning observed in the control by 50% (the inhibitor was not preincubated with mushroom polyphenoloxidase prior to addition of the substrate).
[c] Standard deviation.
[d] N = Not detectable.

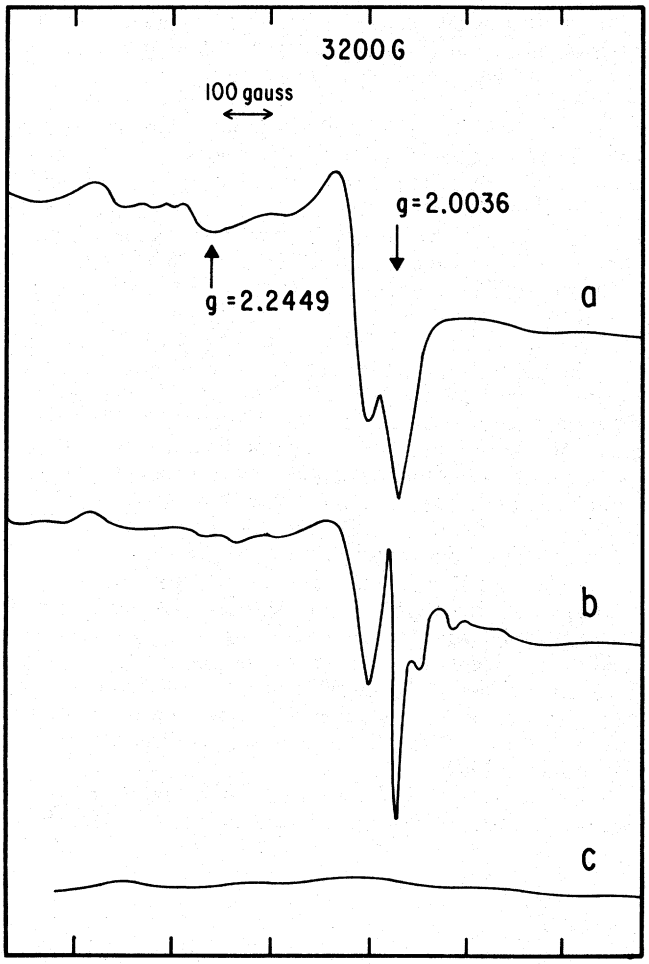


*Fig. 2—EPR spectra of polyphenol oxidase in the presence or absence of ascorbic acid. Spectra (a) PPO (10 mg/mL) in sodium phosphate buffer, 50 mM pH 6.8 (b) the same conditions as spectrum (a) but with the addition of 10 mM of sodium ascorbate (c) sodium phosphate buffer (50 mM, pH 6.8) alone.*

mechanism for that aspect of the inactivation of ascorbic acid on PPO due to the reduction of $Cu^{++}$ to $Cu^{+}$. In addition, the effects of various ascorbic acid derivatives such as dehydroAA, isoAA, AA-2-$PO_4$, and AA-2-$SO_4$ on PPO activity were also examined. The effectiveness of these compounds on inhibition of PPO was determined, and the possibility of conversion of AA-2-$PO_4$ and AA-2-$SO_4$ to AA for controlling PPO activity was investigated. The results from this study might lead to the use of these compounds in the control of enzymatic browning in fresh fruits and vegetables.

## MATERIALS & METHODS

MUSHROOM PPO, acid phosphatase and sulfatase, L-DOPA (dihydroxyphenylalanine), dehydroascorbic acid and isoascorbic acid, and ascorbic-2-sulfate were from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid was from Aldrich Chemical Co. (Milwaukee, WI). AA-2-$PO_4$ was a gift from Dr. Paul A. Seib, Dept. of Grain Science & Industry, Kansas State Univ., Manhattan, KS.

### PPO assay

A spectrophotometric method was used to determine the PPO activity (Hsu et al., 1984). A standard assay was carried out on a 2 mL aliquot of reaction medium (saturated with oxygen) containing 0.25 mM DOPA and mushroom PPO (20 μg) in 50 mM of sodium phosphate (pH 6.8). The reaction was initiated by the addition of substrate and carried out in Beckmann Model 26 spectrophotometer which automatically recorded the absorbance at 419 nm as a function of time. All data were the average of at least three experiments.

### Enzyme-inhibitor incubation

If the inhibitors (AA, dehydroAA, isoAA, AA-2-$PO_4$, and AA-2-$SO_4$) were not preincubated with PPO, the inhibitors were added to PPO prior to the addition of substrate; the reaction was then carried out as described above. The concentration of each inhibitor is indicated in the legends of the Figures and Tables.

To study the effect of preincubation of inhibitors on PPO activity, the inhibitors were incubated with the enzyme in the absence of substrate in a constant shaking bath (Blue M Electric Co., Blue Island, IL). At appropriate time intervals, aliquots were withdrawn, and substrate was added to start the reaction as described above. The absorbance change at 419 nm of the aliquot obtained 10 min after the addition of substrate was used to determine the PPO activity.

### Digestion of AA-2-$PO_4$ or AA-2-$SO_4$ with acid phosphatase or sulfatase

AA-2-$PO_4$ (50 μmole) was added to the sodium acetate (0.15M, pH 5.0) or sodium phosphate buffer (0.05M, pH 6.8) containing 0.6 to 1 units of acid phosphatase. The mixture was incubated for various time intervals (from 0 to 3 hr) at either 25°C or 37°C. The amount of ascorbic acid formed was determined by the method of Howritz (1965). In the case of sulfatase digestion of AA-2-$SO_4$, the same incubation condition was performed as described above, except the enzyme (sulfatase) was increased to 15 units.

### Electron paramagnetic resonance spectroscopy (EPR)

Measurements of cupric ion ($Cu^{2+}$) (Himmelright et al., 1980) concentration were made on aqueous solutions at subfreezing temperature using an electron paramagnetic resonance spectrometer (Model E 109B, Varian Associates) equipped with a frequency counter and a data acquisition system. The thermostatic system for controlling the sample temperature range 77K to room temperature or higher was incorporated in the resonator of the spectrometer.

## RESULTS & DISCUSSION

THE EFFECT of AA on the browning of an aqueous solution containing DOPA and mushroom PPO depended on the concentration of AA. When all components were mixed together at time zero, the lag time preceding the development of colors became longer with increasing concentrations of AA and the degree of browning at the end of the 15 min period decreased as AA increased. Similarly, the lag time increased with increasing ascorbic acid concentrations (Golan-Goldhirsh and Whitaker, 1984). At 0.5 mM, AA completely suppressed the

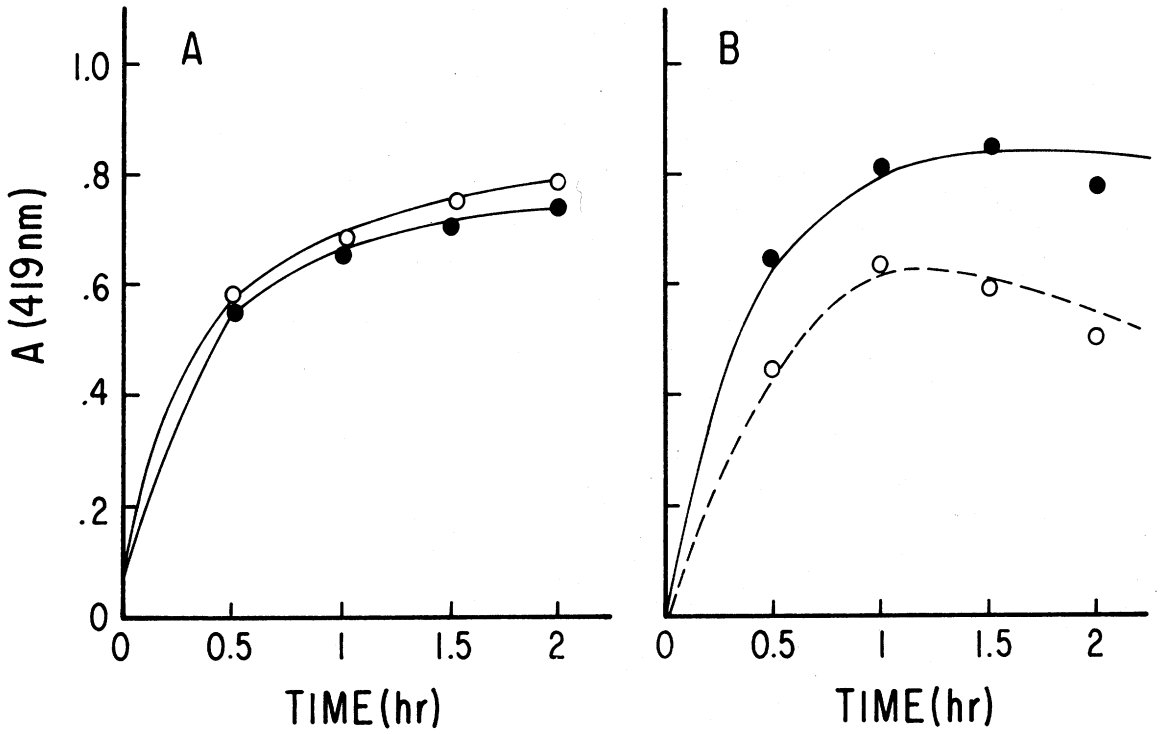


*Fig. 3—Effect of (A) sulfatase (B) phosphatase on the inhibition of mushroom PPO activity by AA-2-phosphate and AA-2-sulfate. The control experiments (●—●) contained mushroom PPO (20 μg), 0.5 mM of either AA-2-$PO_4$ or AA-2-$SO_4$ with DOPA (0.5 mM). The effect of sulfatase (○—○) or phosphatase (○- - -○) on mushroom PPO was determined by the addition of 2 units of sulfatase or phosphatase to the control experiment at zero time of incubation.*

development of brown pigments for several hours. The effectiveness of AA on inhibition of PPO was compared to four related derivatives by incubating 0.25 mM solutions of each inhibitor with 0.50 mM DOPA and mushroom PPO (Fig. 1), AA and isoAA were equally effective in retarding the development of brown pigments. DehydroAA was less effective than AA. AA-2-$SO_4$ and AA-2-$PO_4$ had no measurable effect in this simple system, even when concentrations were increased to 5 mM. This was anticipated since neither phosphatase nor sulfatase which hydrolyze the derivatives to AA were present. The effectiveness of AA, isoAA and dehydroAA in retarding browning was relatively constant for at least 8 to 10 hr. Neither AA-2-$SO_4$ nor AA-2-$PO_4$ retarded the development of browning as compared to the control. AA and isoAA initially were more effective than dehydroAA in suppressing browning, but after 1 hr dehydroAA was slightly more effective and slightly more stable throughout the incubation.

The effect of AA, isoAA and dehydroAA on mushroom PPO was not completely reversible. PPO incubated with these compounds less than 1 hr retained up to 90% of its original activity as determined by precipitating the PPO with ammonium sulfate to remove it from the inhibitors and then assaying for activity in the absence of inhibitors. However, 50% or more of the activity was lost when PPO was incubated with these compounds for 8 to 10 hr. In contrast to our results, Ponting (1954) indicated that PPO isolated from either apple or mushroom was reversibly inactivated by ascorbic acid.

The effects of pretreatment with AA, dehydroAA, and isoAA on PPO, compared to that of the system without preincubation, are shown in Table 1. AA was the most effective inhibitor of browning when added at the start of the incubation period, ($I_{1/2}$, 0.04 mM) while IsoAA and dehydroAA were less effective in reducing the activity of PPO ($I_{1/2}$, 0.25 mM and 7.5 mM, respectively). When all the inhibitors were preincubated with PPO, AA-2-$SO_4$ and AA-2-$PO_4$ had no effect on subsequent activity of the enzyme. All of the other compounds reduced the activity of PPO by about 50% after 2 or 3 hr preincubation with dehydroAA being slightly more effective. Under similar experimental conditions, 5 mM AA was found to inhibit 50% of the PPO activity after 130 min (Golan-Goldhirsh and Whitaker, 1984).

The treatment effect of AA on the activity of mushroom PPO has also been observed by Golan-Goldhirsh and Whitaker (1984). These authors suggested that AA had a peculiar effect on PPO and proposed that AA changed to a more reactive species during the early stage of incubation. In this study, AA has a direct effect on PPO by reducing $Cu^{++}$ of PPO to $Cu^{+}$ (Fig. 2). Figure 2a shows electron paramagnetic resonance spectra of polyphenol oxidase with hyperfine splitting of approximately 180 gauss. There were two components in the g = 2.00 region similar to that reported by Bouchilloux et al. (1963). In their study, both cupric and cuprous forms of PPO were detected by EPR. However, the reduction of cupric to cuprous form by ascorbate was not addressed by EPR. Preliminary experiments following changing absorbance of 2′,2′-biquinoline suggested that ascorbate may be able to reduce the copper of PPO. These data indicated that either more than one type of $Cu^{2+}$ binding site was present or the presence of a free radical. When sodium ascorbate was included in the enzyme solution, the EPR spectra also gave cupric copper signals but in decreased spin concentration as depicted in Fig. 2b. The sharp resonance in the g = 2.00 region increased in intensity indicating the possible formation of a free radical such as free electron; the g value is 2.0023. Our data indicated that $Cu^{2+}$, associated with the enzyme, was reduced upon addition of ascorbate.

As shown before (Fig. 1), AA-2-$PO_4$ and AA-2-$SO_4$ were not effective in retarding browning in the simple model system containing DOPA and mushroom PPO. These derivatives of AA were formulated with the expectation that they would not be active themselves but that they might provide a pool of AA that could be released gradually in a raw fruit or vegetable product that contained the necessary phosphatase or sulfatase to cleave the phosphate or sulfate group to yield AA. Among all the conditions tested at various levels of pH and temperature, the optimal condition for AA-2-$PO_4$ conversion to AA

was pH 6.8 and 25°C. Approximately 2% of AA-2-$PO_4$ was converted to AA after 2 hr of incubation. However, under similar conditions sulfatase was unable to convert AA-2-$SO_4$ to AA. Even with sulfatase present at a rather high concentration relative to that of phosphatase, only minimal conversion of AA-2-$SO_4$ to AA was observed under the optimal conditions (pH 5.0, and 25°C). These data indicated that AA-2-$SO_4$ was a poor substrate for sulfatase. The effect of adding phosphatase or sulfatase to systems containing DOPA, PPO and either AA-2-$PO_4$ or AA-2-$SO_4$, is shown in Fig. 3. In the presence of phosphatase, AA-2-$PO_4$ was able to reduce the formation of brown pigments, presumably through the hydrolysis of AA-2-$PO_4$ to yield AA. Reduction of browning in systems containing sulfatase and AA-2-$SO_4$ was not observed. This is consistent with our finding that AA-2-$SO_4$ was a poor substrate for sulfatase.

The data reported here indicated that ascorbic acid inhibited mushroom PPO by a mechanism not previously suggested. In addition, we also presented the possible application of AA-2-$PO_4$ to replace AA as an inhibitor of browning in foods.

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